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14. ABSTRACT Around 35% of Persian Gulf War soldiers suffer from the Gulf War Illness (GWI). Exposure to organophosphates (OP) is thought to underlie GWI. Disruptions in Ca ²⁺ homeostasis are found in many neurological conditions, but has not been studied in GWI. Male Sprague-Dawley rats were exposed to various diisopropyl fluorophosphate (DFP) dose to approximate GW OP exposures. We observed depressive symptoms and cognitive deficits at 3-mos post DFP exposures. At this time-point, acutely isolated CA1 neurons from GWI rats manifested [Ca ²⁺] _i , that were significantly higher than [Ca ²⁺] _i in neurons from age-matched control rats. Analysis of the population distributions of [Ca ²⁺] _i revealed a significant right-ward population shift towards higher Ca ²⁺ level in DFP rats. Analysis of protein expression for the components of Ca ²⁺ -induced Ca ²⁺ -release (CICR) machinery revealed a significant upregulation in the levels of phosphorylated ryanodine receptor, and signaling enzymes PKA and PLC γ . Treatment with CICR antagonists significantly dropped elevated [Ca ²⁺] _i in DFP neurons and also improved performance on neurobehavioral tests. Since Ca ²⁺ is a major second messenger molecule, sustained increase in its levels could activate multiple signaling cascades, and alter expression of proteins involved in synaptic plasticity, which could underlie the chronic morbidity following DFP exposures.					
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1. Introduction

Around 35% of Persian Gulf War soldiers exhibit chronic multi-symptom illnesses also known as the Gulf War Illness (GWI). Amongst many causative factors, exposure to low-levels of Sarin has been strongly implicated for expression of GWI. Despite treatment recommendations, GWI veterans continue to suffer from anxiety, depression, and memory impairments. There are several confounding factors attributed to the development of GWI, and after reviewing all of the available data, the Research Advisory Committee on Gulf War Veterans' Illnesses has strongly implicated exposure to organophosphates (OPs) as one of the leading cause for GWI [1,2]. Diisopropyl fluorophosphate (DFP) is an OP compound that is used in civilian laboratories as a surrogate nerve gas agent [3,4]. We have used this agent to mimic OP exposure during Gulf War deployment and have observed depressive symptoms and cognitive deficits in rats exposed to repeated, low-dose DFP and single, high-dose DFP exposure [3,5]. Here we investigate a molecular basis for the development of GWI neurological morbidities in our DFP rodent model.

Calcium is a major second messenger and plays a vital role in cellular signaling, in developing neuronal plasticity which affects behavior, and memory [6,7]. Thus, the levels of Ca^{2+} are tightly regulated by an intricate system of ion-channels, buffers, pumps and intracellular stores (ER). Brief elevations in Ca^{2+} levels are critical to cellular communication and long-term potentiation (learning and memory consolidation). However, our research and that of other investigators have demonstrated that sustained Ca^{2+} elevations particularly in the hippocampal region are detrimental to the cell and are implicated in many neurological disorders including Alzheimer's disease [8], Parkinson's disease [9], traumatic brain injury [10], aging [11], epilepsy [12] and stroke [13]. These neurological conditions are typically associated with cognitive deficits and other bio-behavioral disorders. The hippocampus plays a major role in the limbic system, is essential in memory functioning [14] and plays a major role in pathophysiology of depression [15]. Studies have shown hippocampal dysfunction in Gulf War veterans using both imaging and neuropsychological testing [16-18]. OP-based animal models of GWI have also demonstrated hippocampal and striatal neuronal loss, inflammation, and reduced synaptic transmission underlying the expression of anxiety, mood and memory deficits [19-23]. Thus, hippocampus is an important brain area to investigate in GWI.

There has been recent evidence that Ca^{2+} -induced Ca^{2+} release (CICR), which principally consists of the inositol-trisphosphate receptor (IP_3R) and the ryanodine receptor (RyR), plays a distinct role in memory processing and disease state [7,24-26]. The RyR -dependent Ca^{2+} release appears to aid the consolidation of labile memory into a persistent long-term memory trace while IP_3Rs are required during the formation of long-term memory [7]. The RyR can be activated via multiple mechanisms including the PKA-dependent phosphorylation [27,28]. A permanent activation results in the development of "leaky" RyRs that raise $[\text{Ca}^{2+}]_i$ [27,29]. These phosphorylated RyRs have been implicated in stress-induced cognitive dysfunction [30]. Indeed, it has been recently shown that the RyR antagonist dantrolene significantly improves cognition in a murine model of Alzheimer's disease [31]. Similarly, pharmacological blockade of the intracellular Ca^{2+} release using both the IP_3R and RyR antagonist has been demonstrated to produce anti-depressant effect in forced swim test [32], a widely used rodent model of depression. Furthermore, knockdown of RyR subtypes in the brain also exhibited an anti-depressant effect [33]. Inhibition of IP_3R and $\text{PLC}\gamma$, which is responsible for producing IP_3 [34], has been reported to produce anti-depressant effect in rodents [35]. Moreover, there is new evidence that levetiracetam, which we have reported to inhibit both the IP_3 and RyR mediated

Ca²⁺ release in hippocampal neurons [36], also produces an anti-depressant effect in forced swim test [37], has an anxiolytic profile in the elevated plus maze test [38], and improves memory following traumatic brain injury in rats [39]. We have recently shown that the CICR system in hippocampal neurons was responsible for maintaining the long-lasting Ca²⁺ plateau after brain injury such that treatment with the CICR RyR antagonist, dantrolene, abolished the Ca²⁺ plateau and prevented the development of spontaneous recurrent epileptiform discharges in a hippocampal neuronal culture model of epilepsy [40]. Thus, there is mounting evidence that the hippocampal CICR Ca²⁺ signaling system is critical in mood and memory processing and that disturbance in this cascade produces depressive symptoms and memory impairments, and inhibiting this system with pharmacological or genetic manipulation affords relief from the symptoms of the affective disorders. At present, the role of CICR signaling system in the development of depression and cognitive impairments in GWI is unknown and will be investigated in these studies using a rodent model of GWI developed in our laboratory [3].

2. Keywords

Gulf War Illness, Organophosphate, diisopropyl fluorophosphate (DFP), neurological morbidities, Sprague-Dawley rats, Calcium imaging, Fura-2, Calcium-induced Calcium Release, Dantrolene, Levetiracetam

3. Accomplishments:

The following lists the accomplishments from our project during the year-2 (2015-2016).

3.1 What were the major goals of the project?

The major goals of the projects for year 2 are highlighted in the SOW below. We have accomplished all these goals. A detailed description of each of these milestones is in section 3.2

Specific Aim 2A: To identify molecular changes particularly in the components of CICR systems responsible for chronic neuronal plasticity	Months	% Completed
Subtask 1: Assessment of GWI morbidities at 3-m following DFP exposure (0.1 mg/kg, s.c., 10-days, these animals will be used in subtask 2 and 3)	11-13	100%
Subtask 2: Measurement of [Ca ²⁺] _i and CICR activity	11-13	90%
Subtask 3: Harvesting of proteins and running Western blotting experiments for identifying alterations in the levels of RyR and IP ₃ -R	11-13	100%
Subtask 4: Assessment of GWI morbidities at 3-m following DFP exposure (0.5 mg/kg, s.c., 5-days, these animals will be used in subtask 5 and 6)	14-16	100%
Subtask 5: Measurement of [Ca ²⁺] _i and CICR activity	14-16	90%
Subtask 6: Harvesting of proteins and running western blotting experiments for identifying alterations in the levels of RyR and IP ₃ -R	14-16	100%

Subtask 7: Assessment of GWI morbidities at 3-m following DFP exposure (4 mg/kg, s.c., 1-day, these rats will be used in subtask 8, 9	17-19	100%
Subtask 8: Measurement of $[Ca^{2+}]_i$ and CICR activity	17-19	90%
Subtask 9: Harvesting of proteins and running Western blotting experiments for identifying alterations in the levels of RyR and IP ₃ -R	17-19	100%
Specific Aim 3A: Elucidate effects of drugs that target the altered components of CICR systems responsible for chronic behavioral morbidity	Months	% Completed
Subtask 11: Study the effect of CICR-targeted drugs (dantrolene, levetiracetam, U-73122 and H-89) on depression and memory in rats exposed to DFP levels at 3-months post exposure	20-22	50% (U-73122 & H-89 to be tested with 12-m grp)
DATA ANALYSIS	22-23	100%

3.2 What was accomplished under these goals?

We measured intracellular calcium levels, studied handling of the intracellular calcium-induced calcium release (CICR) mechanisms, and estimated protein levels of CICR components in GWI rats displaying anxiety, depression and cognitive deficits. Effects of drugs targeting the CICR components in relieving GWI neurological morbidities were also investigated.

I. DFP exposure

DFP was prepared fresh daily by dissolving in ice-cold phosphate buffered saline just before the exposure. Rats were injected with DFP at three dose levels. Repeated ultra-low dose (0.1 mg/kg, s.c., 1x daily for 10-days), repeated low dose (0.5 mg/kg, s.c., 1x daily for 5-days) and single high-dose (4 mg/kg, s.c. 1x). Rats at the high-dose levels were treated with atropine to temper cholinergic crises, 2-PAM to improve survival, and diazepam to stop seizures and improve outcome. Control rats received DFP vehicle injections for the same period. Animal health including weight measurements were assessed every day during the exposure and for the next seven days following the end of DFP injections.

II. Estimation of hippocampal intra-neuronal Ca^{2+} levels: In these experiments, control and GWI rats at various time-points post-DFP exposures (0.1-4 mg/kg, s.c.) were utilized to estimate intracellular Ca^{2+} levels. Briefly, rats were decapitated, brains removed and hippocampal slices obtained on a vibrotome. Following enzymatic treatment, hippocampus was removed and triturated to generate a neuronal suspension. Calcium levels were measured using microfluorimetry. These steps are described below:

II a. Isolation of Hippocampal CA1 Neurons and Loading with Fura-2

Acute isolation of CA1 hippocampal neurons was performed by established procedures routinely used in our laboratory [4,41]. Animals were anesthetized with isoflurane and decapitated. Brains were rapidly dissected and placed in 4°C oxygenated (95% O₂/5% CO₂) artificial cerebrospinal

fluid (aCSF) consisting of (in mM): 201.5 sucrose, 10 glucose, 1.25 NaH₂PO₄, 26 NaHCO₃, 3 KCl, 7 MgCl₂, and 0.2 CaCl₂. MK-801 (1 μ M) was added to all solutions to increase cell viability and was removed 15 min prior to imaging. Hippocampal slices (450 μ m) were cut on a vibrating microtome (Leica Microsystems, Wetzlar, Germany) and then equilibrated for 10 min at 34°C in a piperazine-N,N'-bis[2-ethanesulfonic acid] (PIPES)-aCSF solution containing (in mM): 120 NaCl, 25 glucose, 20 PIPES, 5 KCl, 7 MgCl₂, and 0.1 CaCl₂. Slices were then treated with 8 mg/ml protease in PIPES-aCSF for 6 min at 34°C and rinsed. Enzyme treated slices were visualized on a dissecting microscope to excise the CA1 hippocampal layer which was then triturated with a series of Pasteur pipettes of decreasing diameter in cold (4°C) PIPES-aCSF solution containing 1 μ M Fura-2 AM (Invitrogen, Carlsbad, CA). The cell suspension was placed in the middle of 2 well glass-bottomed chambers (Nunc, Thermo Scientific). These glass chambers were previously treated overnight with 0.05 mg/ml poly-L-lysine followed by multiple rinses with distilled water and then further treated with Cell-Tak™ (BD-Biosciences, San Jose, CA) biocompatible cellular adhesive (3.5 μ g/cm²) for 30-min, rinsed and air-dried. Neuronal suspension placed in the center of adhesive coated dishes when settled firmly adhered to the bottom. This technique simplified further manipulations on the dissociated neurons. Plates were then incubated at 37°C in a 5% CO₂/95% air atmosphere for 45 min. Fura-2 was washed off with PIPES-aCSF and plates were incubated an additional 15 min to allow for complete cleavage of the AM moiety from Fura-2.

II b. Measurement of [Ca²⁺]_i

Fura-2 loaded cells were transferred to a 37°C heated stage (Harvard Apparatus, Hollington, MA) on an Olympus IX-70 inverted microscope coupled to a fluorescence imaging system (Olympus America, Center Valley, PA) and subjected to [Ca²⁺]_i measurements by procedures well established in our laboratory [4,41]. All experiments were performed using a 20X, 0.7 N.A. water immersion objective and images were recorded by an ORCA-ER high-speed digital CCD camera (Hamamatsu Photonics K.K., Japan). Fura-2 was excited with a 75 W xenon arc lamp (Olympus America, Center Valley, PA). Ratio images were acquired by alternating excitation wavelengths (340/380 nm) by using a Lambda 10-2 filter wheel (Sutter Instruments Co., Novato, CA) and a Fura filter cube at 510/540 emission with a dichroic at 400 nm. All image acquisition and processing was controlled by a computer connected to the camera and filter wheel using Metafluor Software ver 7.6 (MDS Analytical Technologies, Downingtown, PA). Image pairs were captured every 5s and the images at each wavelength were averaged over 10 frames. Background fluorescence is obtained by imaging a field lacking Fura-2. Hippocampal CA1 neurons were identified based on their distinct morphology. These neurons displayed pyramidal shaped cell body, long axon and dendrites and have been demonstrated to be devoid of immunoreactivity for specific protein markers for interneurons, including parvalbumin, cholecystokinin, vasoactive intestinal peptide, somatostatin, and neuropeptide Y. The process of enzymatic treatment and mechanical trituration can add minimal stress during acute dissociation of neurons. However, we have shown previously that the neurons isolated using these procedures exhibit electrophysiological properties identical neurons in slices or in cultures, are viable, and not apoptotic or necrotic.

II c. Calcium calibration

We performed Ca²⁺ calibration determinations as described previously [4,41,42] to provide estimates of absolute [Ca²⁺]_i concentrations from the 340/380 ratio values. A Ca²⁺ calibration

curve was constructed using solutions of calibrated Ca^{2+} buffers ranging from 0 Ca^{2+} (Ca^{2+} free) to 39 μM Ca^{2+} (Invitrogen, Carlsbad, CA). Values from the calibration curve were used to convert fluorescent ratios to $[\text{Ca}^{2+}]_i$. Final $[\text{Ca}^{2+}]_i$ were calculated from the background corrected 340/380 ratios using the Grynkiewicz equation:

$$[\text{Ca}^{2+}]_i = (\text{K}_d \times \text{Sf}_2 / \text{Sb}_2) \times (\text{R} - \text{R}_{\min}) / (\text{R}_{\max} - \text{R})$$

where R was the 340/380 ratio at any time; R_{\max} was the maximum measured ratio in saturating Ca^{2+} solution (39 μM free Ca^{2+}); R_{\min} was the minimal measured ratio Ca^{2+} free solution; Sf_2 was the absolute value of the corrected 380-nm signal at R_{\min} ; Sb_2 was the absolute value of the corrected 380-nm signal at R_{\max} ; the K_d value for Fura 2 was 224 nM.

III. Identification of molecular changes in intracellular Ca^{2+} handling mechanism: Animals were sacrificed and hippocampal tissue processed for Western blot studies at each time point following DFP exposure using standard procedures established in the literature and routinely performed in this laboratory to measure proteins in control and DFP hippocampi. From each experimental animal, hippocampal homogenates were prepared. Quantitation of the amount of RyR-p, IP3-R, PKA and PLC γ per mg protein in each sample was performed. Antibody specificity was established using blocking peptides and no antibody controls. Internal and external standards to control for loading and sample variability were used. The following describes the procedure for RyR2 protein estimation. Similar protocols were utilized for estimating other protein using specified antibodies with minor variations.

III a. Isolation and homogenization of hippocampal tissue

Control and GWI rats were anesthetized by inhalation of isoflurane until the righting reflex was lost and respiration rate slowed and were then rapidly decapitated. The brain was quickly removed and dissected. Hippocampal tissue was separated from the rest of the brain and flash-frozen in liquid nitrogen. The frozen hippocampus was homogenized using a motorized homogenizer in 750 μL of modified, ice-cold RIPA buffer consisting of: 1% NP-40, 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 1% Triton- X100, as well as, phenylmethanesulphonyl-fluoride (PMSF), and Halt Phosphatase Inhibitor Cocktail (Pierce, Rockford, IL) to inhibit protease and phosphatase activity, respectively. The homogenates were stored at -80°C and protein concentration was measured using the Bradford reagent (BioRad, Hercules, CA) and a spectrophotometer (595 nm).

III b. Western blotting procedures

Following deep isoflurane anesthesia, rats were decapitated and hippocampus was removed and homogenized in 10 volumes of ice cold buffer (50 mM Tris-HCL, 7 mM EGTA, 5 mM EDTA, 0.32 M sucrose, pH 7.5) containing protease and phosphatase inhibitors. Twelve to fifteen micrograms of sample were resolved on a NuPAGE 4-12% Bis-Tris mini gel (Invitrogen, Carlsbad, CA). Resolved proteins were transferred onto PVDF membrane in a Trans-Blot Cell apparatus (Biorad, Hercules, CA) and immuno-staining was carried out for select proteins. Primary antibodies used for Western analysis included: rabbit-anti-CREB (48H2; 1:2K, Cell Signaling Tech., Danvers, MA), rabbit-anti-phospho(ser133)-CREB (1:2K, Cell Signaling Tech.), goat-anti-PKA RII (1:2K, EMD Millipore, Billerica, MA), rabbit-anti-IP3 (1:2K, EMD Millipore), rabbit-anti RyR-2 (1:1K, EMD Millipore), rabbit-anti-phospho(ser2808)-RyR-2 (1:2K, Leeds, United Kingdom), mouse-anti-PLC- γ 1 (clone 10; 1:1K, BD Biosciences, San Jose, CA) and mouse-anti-actin- β (1:20K, Sigma-Aldrich, St. Louis, MO). HRP-conjugated

secondary antibodies used were mouse-anti-goat, goat-anti-rabbit and goat-anti-mouse (1:10K, Santa Cruz Biotech., Dallas, TX). Westerns were incubated in primary antibodies for 2-14 hours, washed and incubated in secondary antibody for 1 hour. Following additional washes, membranes were incubated in ECL reagent (ECLTMPrime, GE Healthcare Lifesciences, Pittsburgh, PA) for 5 min and then exposed to autoradiographic film (Blue-BioExcell®, WorldWide Medical, Bristol, PA). Films were digitized using a high resolution scanner with backlight, and specific bands were analyzed for relative density (ImageJ software, U.S. National Institutes of Health, Bethesda, MD) and normalized against β -actin to adjust for protein loading.

IV. Behavioral screening assays:

Amongst the GWI morbidities, the neurological deficits such as chronic depression, anxiety and memory impairments are predominant ones. To investigate whether treatment with CICR drugs such as dantrolene and levetiracetam would lead to reduction in the expression of GWI psychiatric abnormalities, we conducted a battery of rodent behavioral assays that identify symptoms of depression, anxiety and cognitive deficits at 3-months following various DFP exposures to closely represent the current status of GW veterans. Testing was carried out in a quiet, dimly lit room between 0800 to 1400 hrs. Depression was assessed using the Forced Swim Test (FST), and the Elevated Plus Maze (EPM). Memory function was assessed using the Novel Object Recognition (NOR). These tests were described recently in our paper on GWI model development [3,5]. Dantrolene (10 mg/kg, i.p.) and Levetiracetam (50 mg/kg, i.p.) were administered 30-mins before the behavioral assays.

V. Data analysis

For comparing the distributions of $[Ca^{2+}]_i$ levels a Chi-square test was used. Data were analyzed and graphs plotted using the SigmaPlot 12.5 software (SPSS Inc, Chicago, IL). All the data that passed the normality test was further subjected to t-test. A value of $p < 0.05$ was considered significant for all data analyses.

VI. Results

VI. a Estimations of hippocampal neuronal $[Ca^{2+}]_i$

To investigate if various DFP treatments caused long lasting changes in hippocampal neuronal $[Ca^{2+}]_i$, we measured neuronal $[Ca^{2+}]_i$ at 3-months after DFP exposures using the high affinity, ratiometric Ca^{2+} indicator Fura-2 in acutely isolated hippocampal neurons from control and DFP treated animals. Acutely isolated CA1 hippocampal neurons harvested from 0.1 mg/kg DFP group manifested mean $[Ca^{2+}]_i$ levels of 217.62 ± 22.12 nM, which was not significantly different than $[Ca^{2+}]_i$ levels harvested from age-matched control (208.12 ± 16.12 nM, $p > 0.05$, $n = 7$ animals). CA1 neurons harvested from rats in 0.5 mg/kg DFP group manifested $[Ca^{2+}]_i$ of 399 ± 26 nM, significantly higher than $[Ca^{2+}]_i$ levels harvested from age-matched control ($p < 0.05$, one-way ANOVA, $n = 8$ animals). Similarly, CA1 neurons harvested from rats in 4 mg/kg DFP group manifested $[Ca^{2+}]_i$ of 458 ± 36 nM, significantly ($p < 0.05$, one-way ANOVA, $n = 8$ animals) higher than $[Ca^{2+}]_i$ levels harvested from age-matched control. This data is shown in Fig. 1A.

Analysis of the population distributions of $[Ca^{2+}]_i$ revealed only 2% of age-matched control neurons exhibited $[Ca^{2+}]_i$ greater than 500 nM. In contrast, ~50% neurons isolated from GWI rats exhibited $[Ca^{2+}]_i$ between 250-500 nM and ~18% neurons exhibited $[Ca^{2+}]_i$ greater

than 500 nM, indicating a significant right-ward population shift towards higher $[Ca^{2+}]_i$ concentration range ($p < 0.001$, Chi-square test, $n = 161$ neurons, Fig. 1B). Similarly, approximately 50% neurons from the DFP treated animals had $[Ca^{2+}]_i$ levels greater than 500 nM indicating a shift in the population of neurons to higher Ca^{2+} ratios. This rightward shift in distribution of $[Ca^{2+}]_i$ levels in DFP neurons was significantly different from control neurons ($p < 0.001$, Chi-square test, $n = 150$ neurons, Fig 1C).

Comprehensive behavioral studies conducted in the first year of performance period showed a dose-dependent effect of DFP exposures on behavior. The behavioral tests did not show any deficits in 0.1 mg/kg DFP exposure group while the 0.5 mg/kg and 4 mg/kg DFP exposure group showed significant neurological morbidities. These findings are in line with the observations reported here wherein the 0.1mg/kg DFP group showed Ca^{2+} levels which were not significantly different from control, but the 0.5 mg/kg and 4 mg/kg DFP exposure groups manifested Ca^{2+} levels that were significantly higher than age-matched control rats. Thus, we are presenting data from the 0.5 mg/kg and 4 mg/kg DFP exposure groups for the subsequent sections to improve readability and focus on the significant effects.

VI. b Estimations of protein levels of some of the components of hippocampal CICR system

Western blot analysis of hippocampal homogenates were carried out to evaluate possible alterations in the levels of select Ca^{2+} -regulatory protein targets following exposure to low (0.5 mg/kg) and high (4.0 mg/kg) DFP. Relative densities for the specific protein bands were measured, corrected for protein loading against β -actin and then calculated as a percent of control \pm SEM. Representative western protein blots are shown in Figure 2.

At 3-months following 0.5 mg/kg DFP exposure (Fig. 2 C,D), we observed an increase in levels of CICR receptor proteins p-RyR-2 ($100 \pm 14.2\%$, $124.2 \pm 31.9\%$) and IP₃-R ($100.0 \pm 48.6\%$, $105.8 \pm 43.2\%$). No significant changes were observed in protein levels of signaling enzymes PLC γ 1 ($100.0 \pm 25.5\%$, $71.6 \pm 8.1\%$) and PKA ($100.0 \pm 22.8\%$, $96.4 \pm 19.7\%$). However, a small increase in regulatory protein p-CREB but not CREB was found at this DFP dose level: CREB ($100.0 \pm 22.0\%$, $80.9 \pm 6.1\%$), p-CREB ($100 \pm 11.7\%$, $119.0 \pm 26.5\%$)

At 3-months following exposure to the high dose of 4.0 mg/kg DFP (Fig. 2 A,B), levels for select Ca^{2+} -regulatory protein targets (control%, DFP% respectively) were as follows: p-CREB ($100 \pm 25.4\%$, $634.7 \pm 120.5\%$), p-RyR-2 ($100 \pm 21.0\%$, $179.6 \pm 22.0\%$), PLC γ 1 ($100.0 \pm 16.8\%$, $132.5 \pm 11.2\%$) and PKA ($100.0 \pm 16.1\%$, $160.6 \pm 16.9\%$). This indicates a dose-dependent response of DFP on the protein level expressions of components of CICR signaling mechanisms, which could lead to protracted elevations in hippocampal neuronal $[Ca^{2+}]_i$ following DFP exposures.

VI. c Mechanism for elevated hippocampal neuronal $[Ca^{2+}]_i$ following DFP exposures

We have demonstrated that Status Epilepticus leads to development of sustained neuronal Ca^{2+} elevations [40, 41] that has its origins in Ca^{2+} release from intracellular stores [42]. To investigate the contribution of intracellular CICR receptors in the development of DFP-induced prolonged Ca^{2+} elevations, $[Ca^{2+}]_i$ levels were measured in neurons obtained from rats exposed to 0.5 mg/kg and 4 mg/kg DFP in the presence of RyR blocker dantrolene and RyR/ IP₃ receptors antagonist levetiracetam.

As shown in Fig. 3A, application of dantrolene (50 μ M) to hippocampal neurons isolated from rats 3-months following 0.5mg/kg DFP resulted in a significant drop in $[Ca^{2+}]_i$ from 399 ± 26 (no drug) to 240 ± 11 nM ($p < 0.01$, $n = 6$ rats). We next investigated the effects of

levetiracetam. As shown in Fig. 3A, application of levetiracetam (100 μ M) produced $[Ca^{2+}]_i$ of 251 ± 19 nM that were significantly lower than $[Ca^{2+}]_i$ in neurons isolated from 0.5mg/kg DFP exposed rats ($p < 0.05$, $n = 6$ rats). This represent almost 60% reduction in $[Ca^{2+}]_i$ following dantrolene or levetiracetam application suggesting that IP3Rs and RyRs via the mechanisms of CICR are significantly contributing to maintaining the DFP induced Ca^{2+} elevations.

As shown in Fig. 3B, application of dantrolene (50 μ M) to hippocampal neurons isolated from rats 3-months following high-dose DFP resulted in a significant drop in $[Ca^{2+}]_i$ from 458 ± 36 (no drug) to 304 ± 11 nM ($p < 0.01$, $n = 5$ rats). We next investigated the effects of levetiracetam. As shown in Fig. 3B, application of levetiracetam (100 μ M) produced $[Ca^{2+}]_i$ of 280 ± 24 nM that were significantly lower than $[Ca^{2+}]_i$ in neurons isolated from high-dose DFP rats ($p < 0.05$, $n = 5$ rats).

VI. d Performance on FST

The FST is an effective test in evaluating the presence of a despair-like state in the DFP exposed rats. Repeated, low-dose DFP rats (0.5 mg/kg, 5-days) subjected to FST exhibited increased immobility time of 78.7 ± 11.5 s indicative of a despair-like state. In the presence of dantrolene (10 mg/kg, i.p.) there was a significant reduction in immobility time in 0.5 DFP exposed group (28.7 ± 6.6 s) that was not significantly higher than age matched controls (44.5 ± 3.8 s, $n = 6$, $p < 0.05$, Fig. 4A).

High-dose DFP rats (4 mg/kg, 1-day) subjected to FST also exhibited increased immobility time of 82.05 ± 10.5 s indicative of a despair-like state. In the presence of dantrolene (10 mg/kg, i.p.) there was a significant reduction in immobility time in 4.0 DFP exposed group (26.3 ± 5.5 s) that was not significantly higher than age matched controls (44.5 ± 3.8 s, $n = 6$, $p < 0.05$, Fig. 4B).

While treatment with levetiracetam (50 mg/kg, i.p.) produced reduction in immobility time in the two DFP exposed groups, statistical significance could not be achieved. A higher dose of levetiracetam will be tested for this paradigm.

VI. e Performance on EPM

DFP exposed rats also displayed symptoms of anxiety when subjected to EPM test. Repeated, low-dose DFP rats (0.5 mg/kg, 5-days) displayed increased anxiety as characterized by significantly lower performance in the open arm of the EPM (time in open arm: $16.9 \pm 2.5\%$ in controls vs $5.28 \pm 1.7\%$ in 0.5 DFP exposed rats) indicating the presence of symptoms of anxiety. In the presence of levetiracetam (50 mg/kg, i.p.) a significant ($p < 0.05$, $n = 6$ rats) anxiolytic effect was observed in the 0.5 DFP exposed groups as identified by an improvement in time spent in the open-arm of EPM which was not significantly different from control group (time in open arm: $16.9 \pm 2.5\%$ in controls vs $14.2 \pm 2.7\%$ in 0.5 DFP exposed rats, Fig. 5A).

High-dose DFP rats (4 mg/kg, 1-day) also displayed increased anxiety as characterized by significantly lower performance in the open arm of the EPM (time in open arm: $16.9 \pm 2.5\%$ in controls vs $3.4 \pm 1.9\%$ in 4.0 DFP exposed rats) indicating the presence of symptoms of anxiety. In the presence of levetiracetam (50 mg/kg, i.p.) a significant ($p < 0.05$, $n = 6$ rats) anxiolytic effect was observed in the high-dose DFP exposed groups as identified by an improvement in time spent in the open-arm of EPM which was not significantly different from control group (time in open arm: $16.9 \pm 2.5\%$ in controls vs $13.5 \pm 3.6\%$ in 4.0 DFP exposed rats, Fig. 5B).

Treatment with dantrolene (10 mg/kg, i.p.) did not improve performance on the EPM in both the DFP dose groups.

VI. f Performance on NOR

The NOR test revealed deficits in recognition memory in DFP exposed rats. In the choice phase of NOR, repeated, low-dose DFP rats (0.5 mg/kg, 5-days) spent more time exploring the old object compared to the new object indicating that these rats did not remember the familiar object. These rats exhibited a discrimination ratio of 0.47 ± 0.08 , indicative of impaired recognition memory that was significantly lower compared to age matched control rats (0.86 ± 0.05 , $n = 6$, $p < 0.05$). In the presence of levetiracetam (50 mg/kg, i.p.) and dantrolene (10mg/kg, i.p.) a significant improvement on NOR performance was observed in moderate dose DFP groups (0.69 ± 0.08 and 0.6 ± 0.07 , respectively, Fig. 6A).

In the choice phase of NOR, high-dose DFP exposed rats (4 mg/kg, 1-day) also spent more time exploring the old object compared to the new object indicating that these rats did not remember the familiar object. These rats exhibited a discrimination ratio of 0.36 ± 0.09 , indicative of impaired recognition memory that was significantly lower compared to age matched control rats (0.73 ± 0.04 , $n = 6$, $p < 0.05$). In the presence of levetiracetam (50 mg/kg, i.p.) and dantrolene (10mg/kg, i.p.) a significant improvement on NOR performance was observed in high dose DFP groups (0.65 ± 0.09 and 0.6 ± 0.1 , respectively).

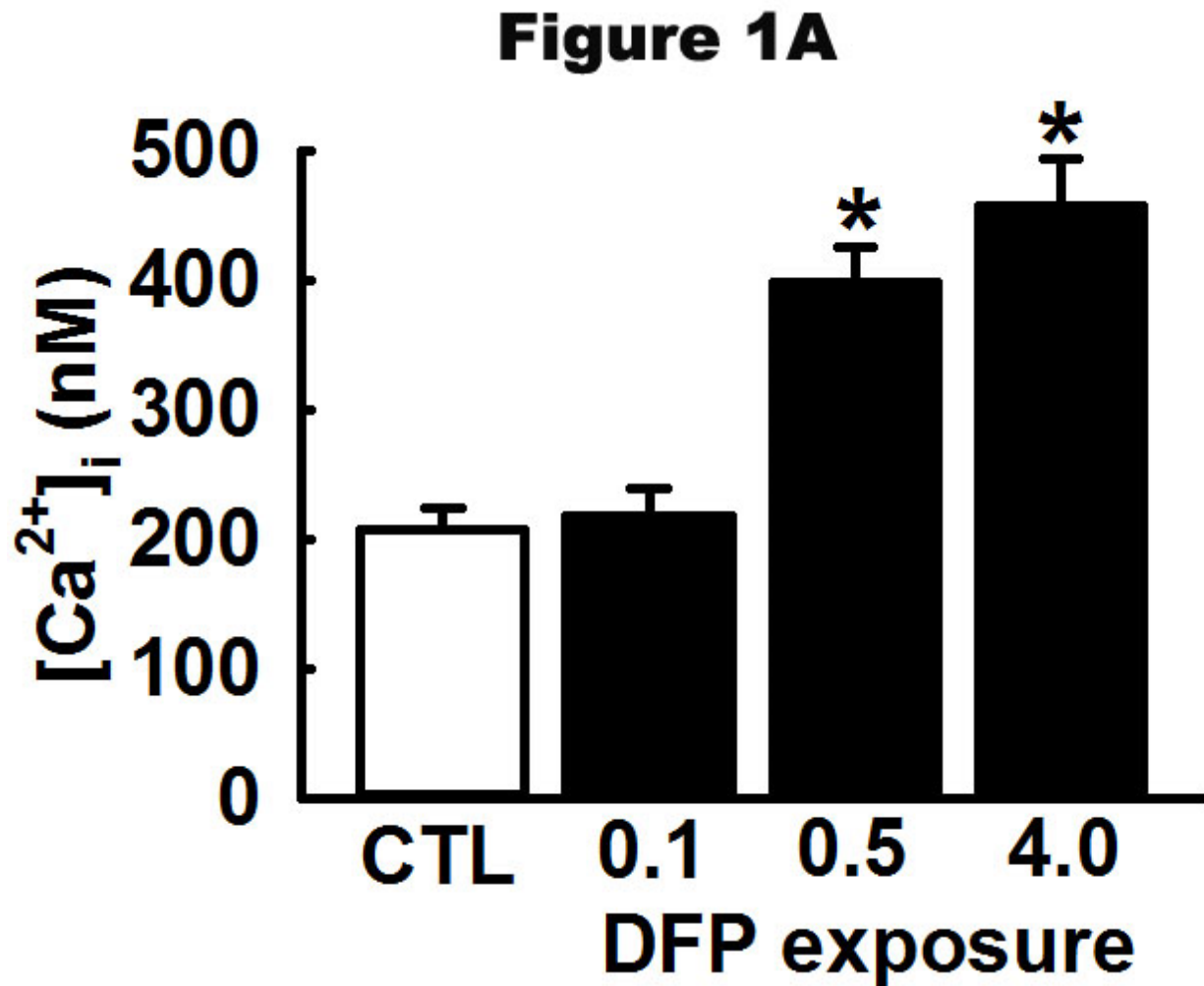


Figure 1A. Elevated $[Ca^{2+}]_i$ in CA1 hippocampal neurons acutely isolated from DFP exposed animals compared to neurons from age-matched control animals. Neuronal Ca^{2+} levels in DFP exposed rats (0.5 and 4 mg/kg group), but not the 0.1 mg/kg DFP group were significantly higher compared to age matched control rats. Data expressed as mean \pm SEM, * p <0.05, t-test, n = 7 rats.

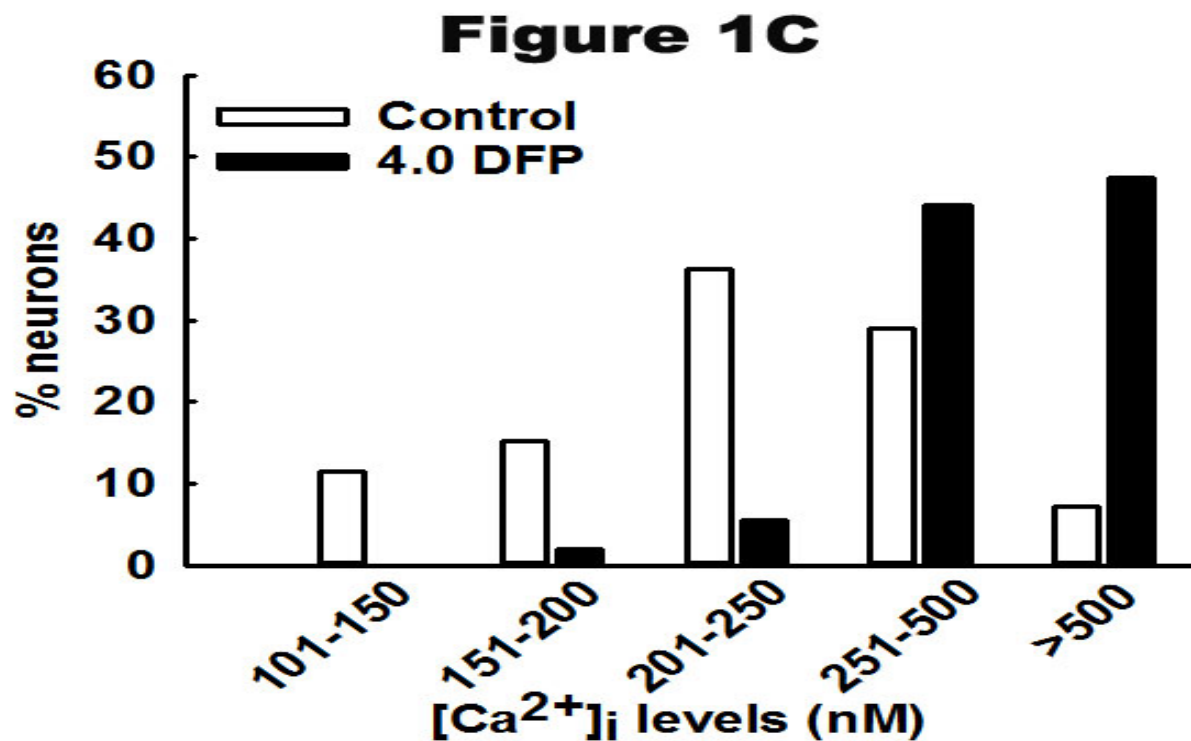
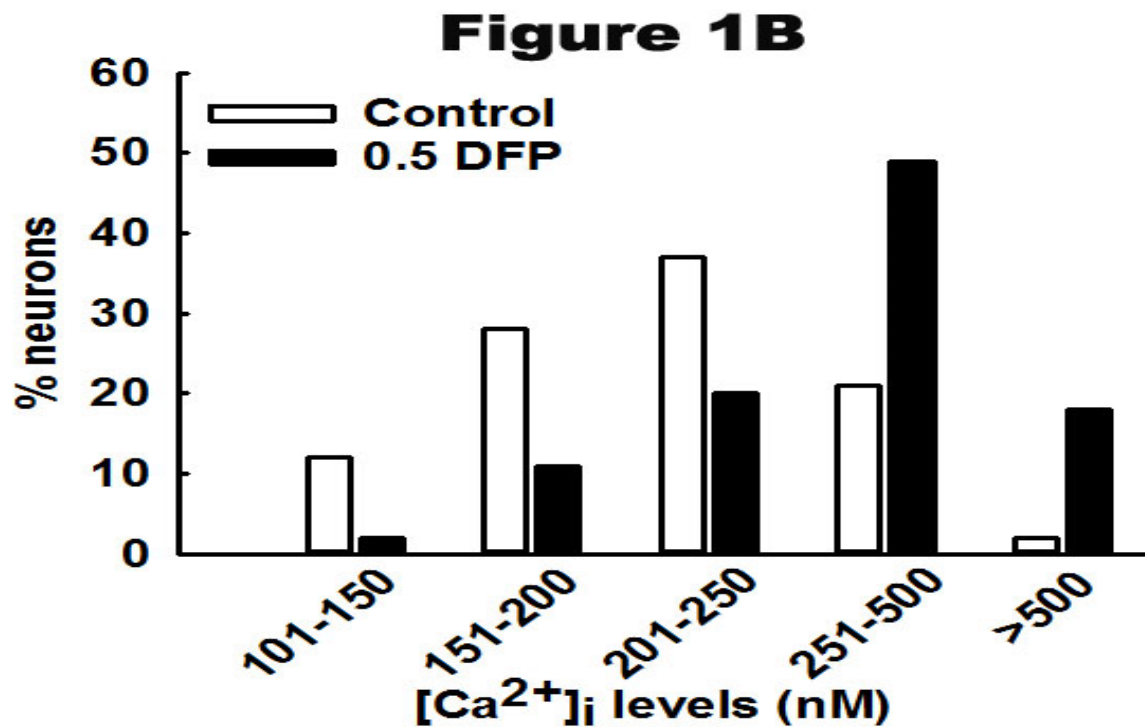


Figure 1B, C. Distribution of $[Ca^{2+}]_i$ for control and DFP-exposed hippocampal neurons. Control neurons demonstrated a normal distribution for $[Ca^{2+}]_i$ with approximately 95% of neurons exhibiting $[Ca^{2+}]_i$ less than 500 nM and only 5% neurons exhibiting very high $[Ca^{2+}]_i$. In contrast, neurons from both the 0.5 mg/kg DFP group (Fig. 1B) and 4 mg/kg DFP group (Fig. 1C) demonstrated a rightward shift towards higher $[Ca^{2+}]_i$ with approximately 50% neurons exhibiting $[Ca^{2+}]_i$ greater than 500 nM (n= 161 and 150 neurons respectively).

Figure 2A

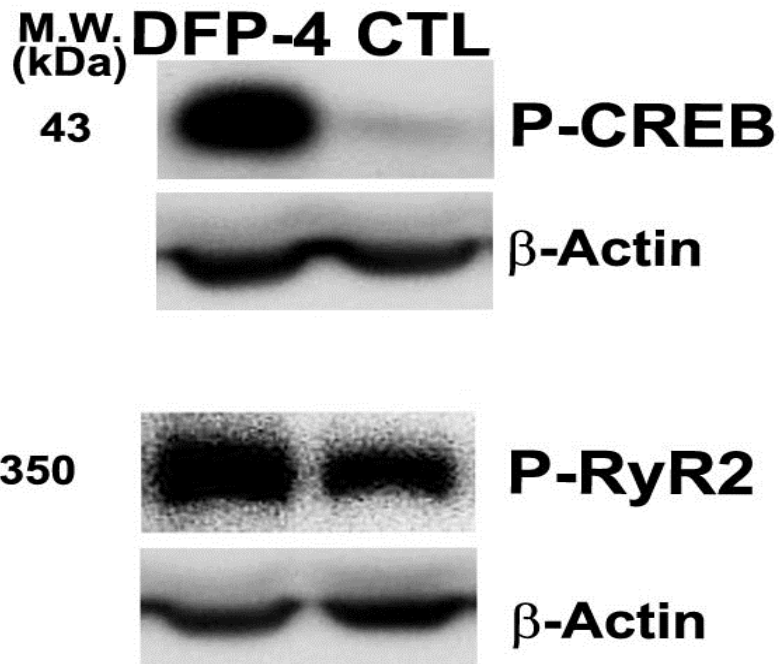


Figure 2B

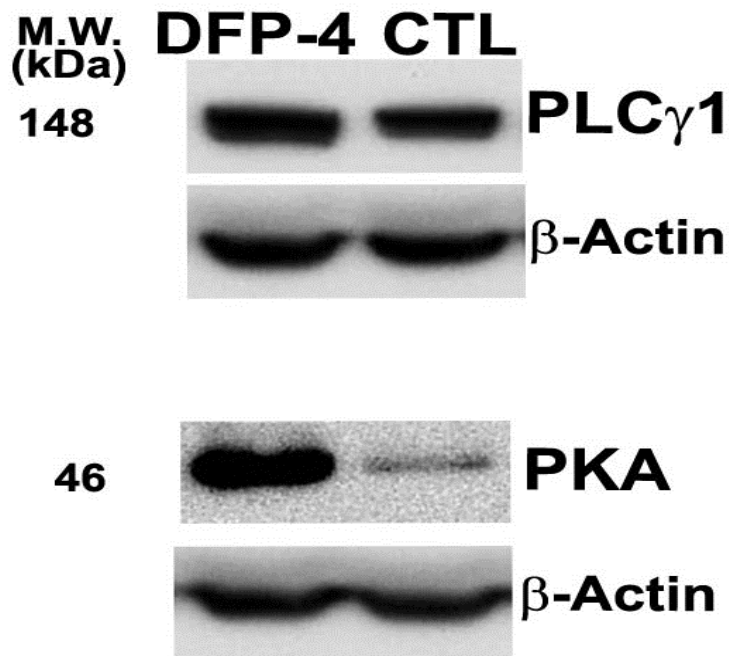


Figure 2A, B. Representative lanes from a Western blot showing increases in p-CREB (top panel-2A) and p-RYR2 (bottom panel-2A) protein levels from control (CTL) and DFP-4 rats. Protein levels of signaling enzymes PLC γ 1 (top panel-2B) and PKA (bottom panel-2B) were also found to be elevated in DFP-4 rats compared to control. β -actin was used as a loading control to confirm equal loading and normalize the optical densities from each protein band.

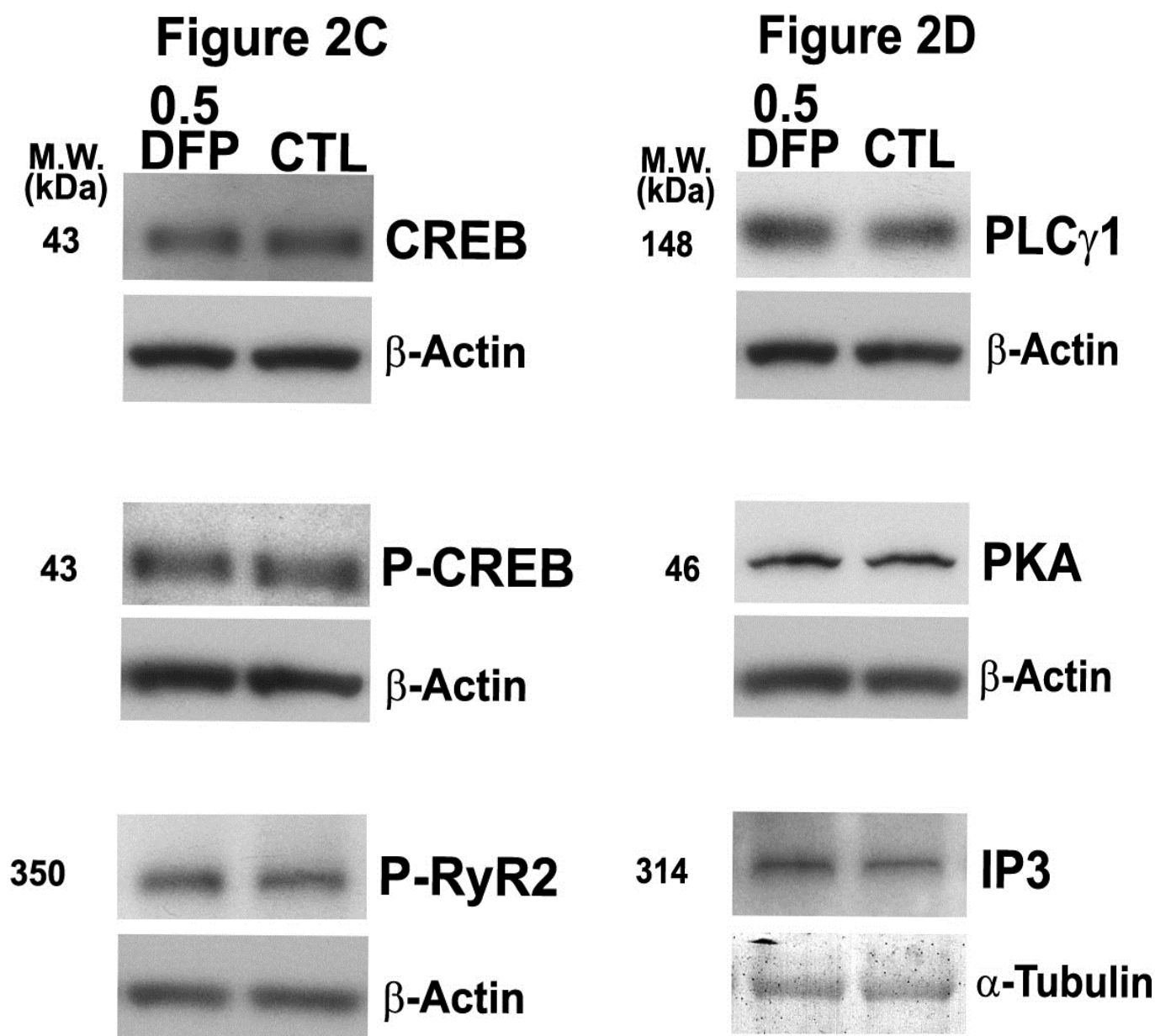


Figure 2C, D. Representative lanes from a Western blot showing no change in CREB (top panel- 2C), increases in p-CREB (middle panel-2C) and p-RYR2 (bottom panel-2C) protein levels from control (CTL) and DFP-0.5 rats. Protein levels of signaling enzymes PLC γ 1 (top panel-2D) and PKA (middle panel-2D) along with IP3-R (bottom panel- 2D) were found to be unchanged in DFP-0.5 rats compared to control (CTL). β -actin was used as a loading control to confirm equal loading and normalize the optical densities from each protein band. α -tubulin was used as a loading control to confirm equal loading and normalize the optical densities from IP3-R protein band.

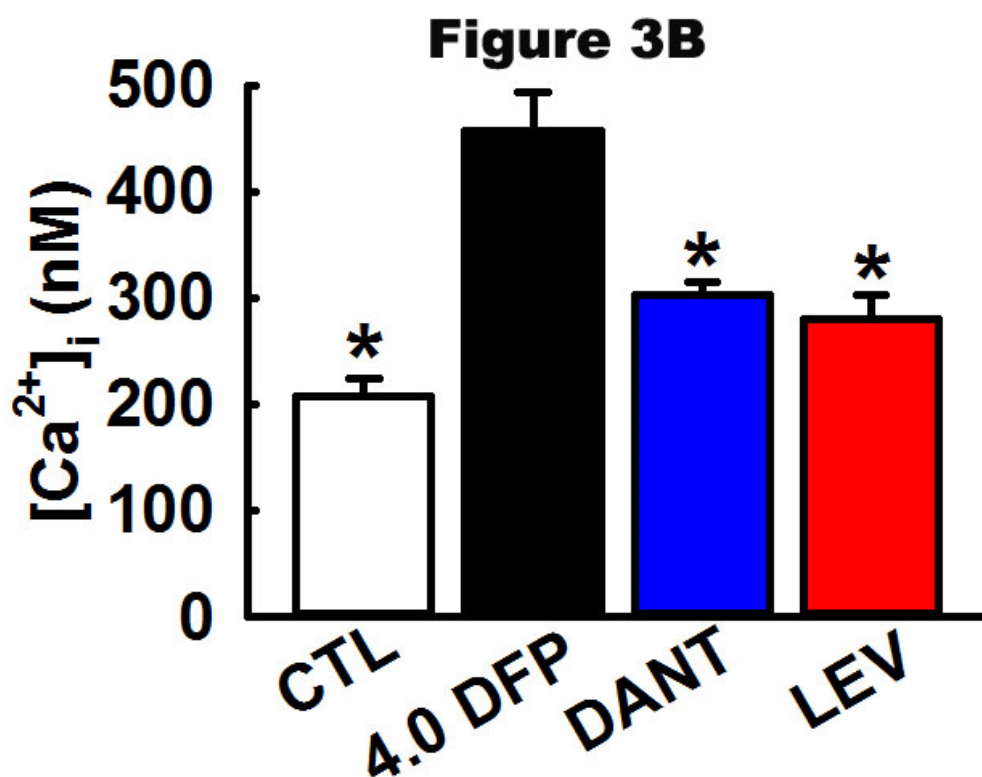
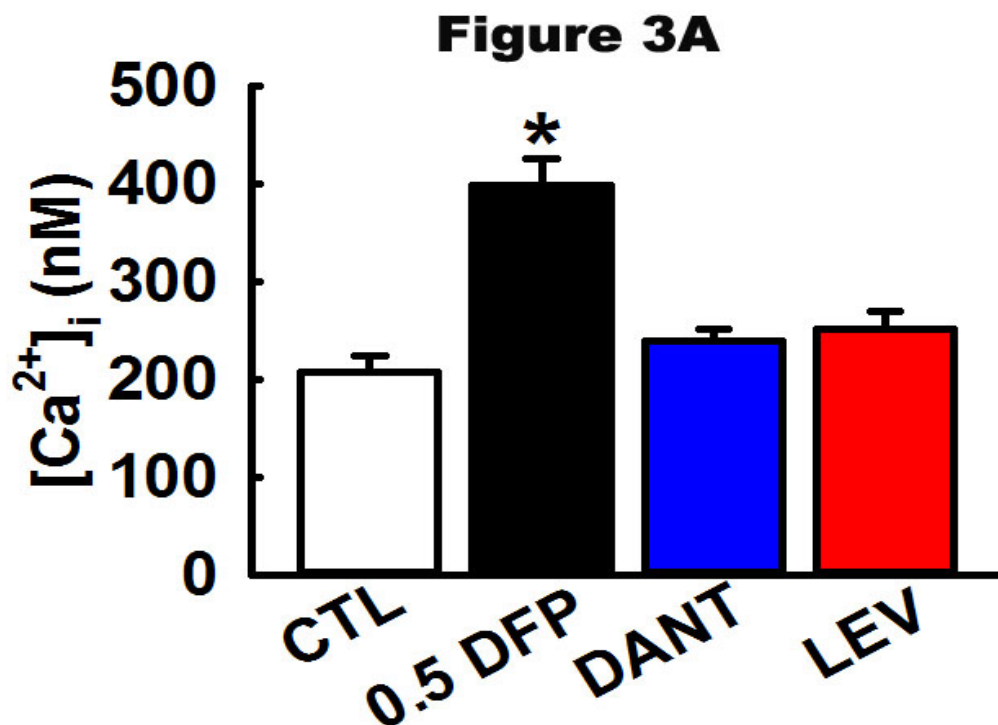


Figure 3A, B. Mechanism of Ca²⁺ plateau following DFP exposure. CA1 hippocampal [Ca²⁺]_i from control (white bar) and DFP exposed rats were isolated at 3-months post exposure (black bar). Application of dantrolene (Dant, 50 μM, blue bar) or levetiracetam (LEV, 100 μM, red bar) caused a significant decrease in elevated Ca²⁺ levels in both the DFP exposed groups. (*p<0.05, compared to DFP, one-way ANOVA, post-hoc Tukey test, n= 5-6 animals for each treatment). Data represented as mean ± SEM.

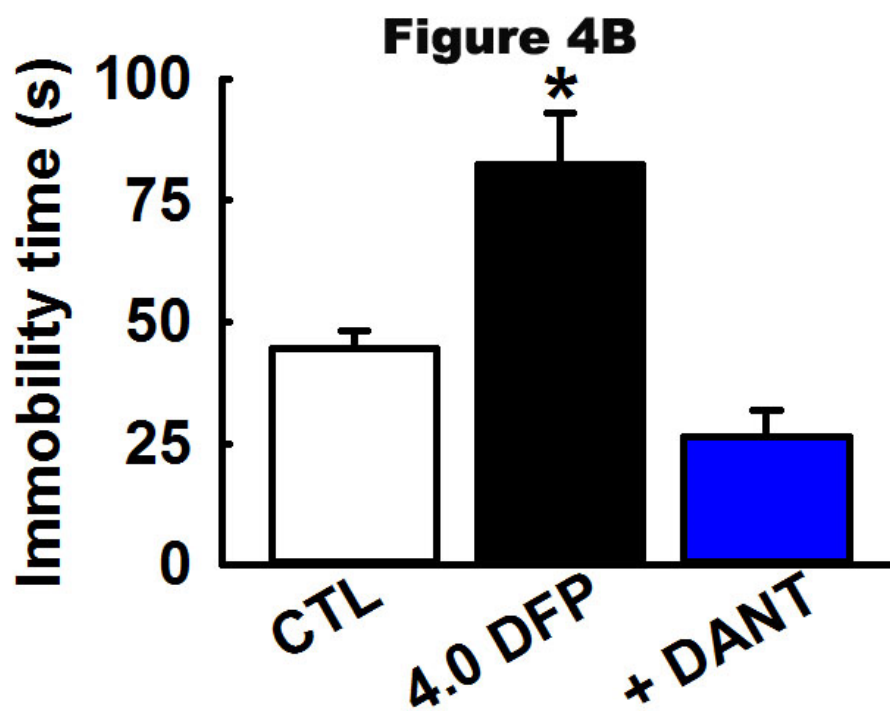
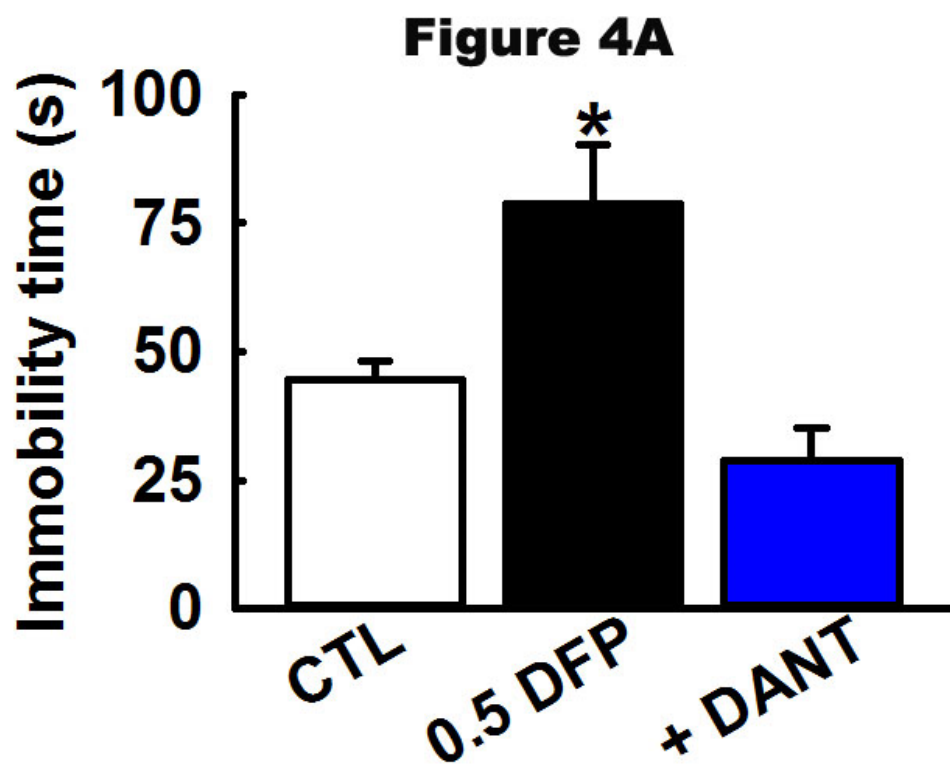


Figure 4A, B. Antidepressant effects of dantrolene. The immobility time in DFP exposed rats (0.5 and 4 mg/kg group) was significantly higher compared to age matched control rats indicative of behavioral despair. Treatment of dantrolene (10 mg/kg, i.p.) significantly lowered the immobility time in both the DFP exposed groups indicative of a robust antidepressant-like effect. Data expressed as mean \pm SEM, * $p < 0.05$, t-test, $n = 6$ rats.

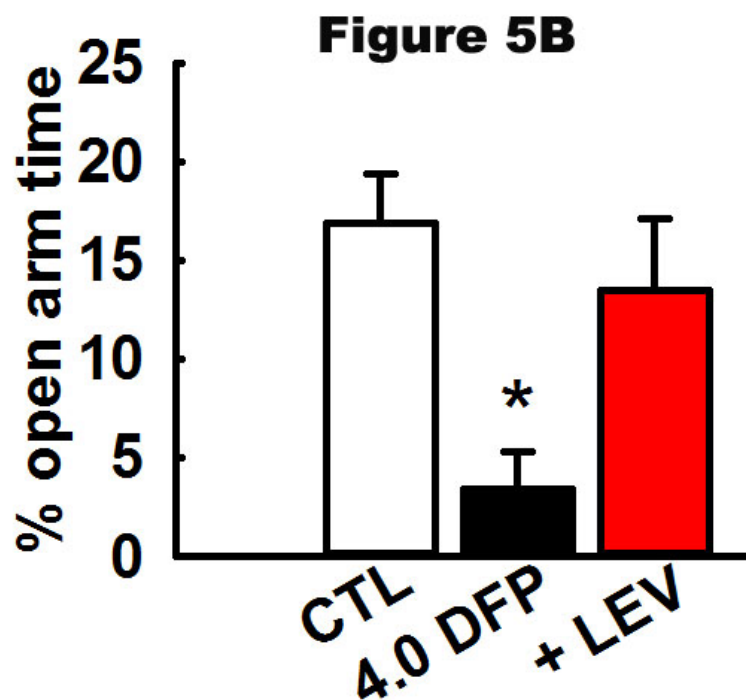
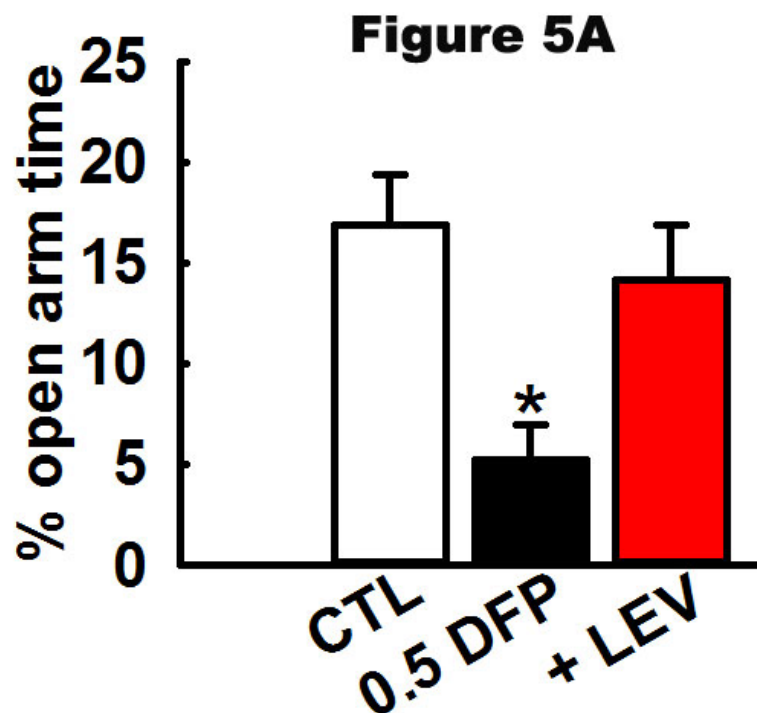


Figure 5A, B. Anxiolytic effects of levetiracetam. At 3-months post DFP exposure, rats at both the dose levels when tested in the EPM task displayed significantly lower open arm time compared to age-matched control rats indicative of increased anxiety. In the presence of levetiracetam (50 mg/kg, i.p.), rats in the both the DFP dose exposure showed a significantly increased exploration of the open-arm suggesting an anxiolytic effect of levetiracetam treatment. Data expressed as mean \pm SEM, * p <0.05, t-test, n = 6 rats.

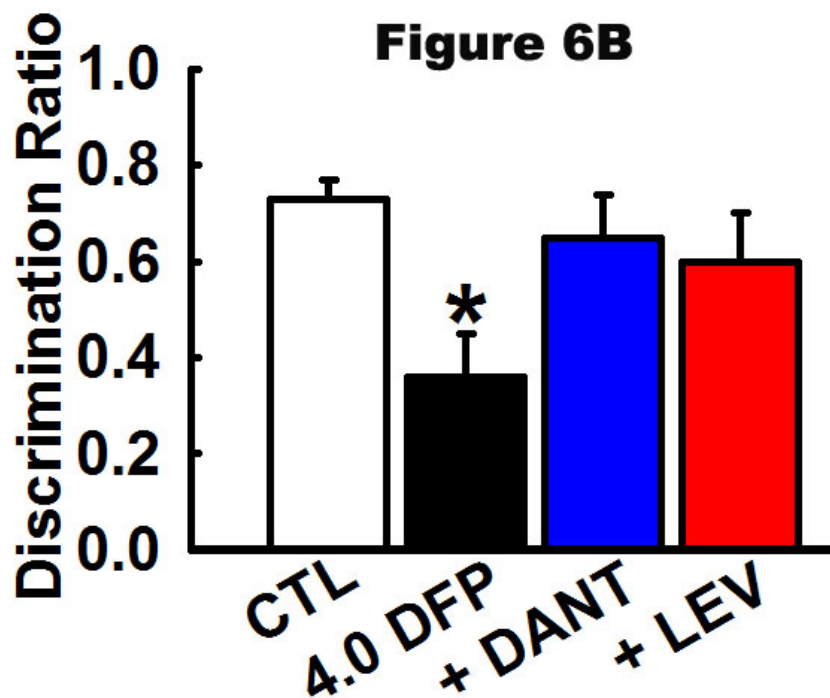
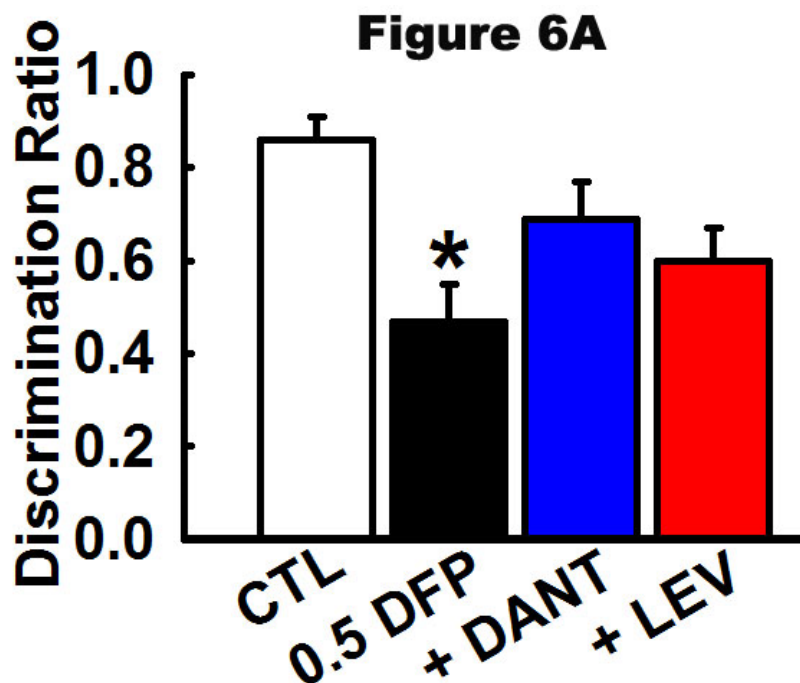


Figure 6A, B. DFP rats (0.5 and 4 mg/kg, s.c.) exhibited a significantly lower discrimination ratio on the NOR test indicative of impaired recognition memory compared to a higher discrimination ratio observed in age matched control rats. In the presence of dantrolene (10 mg/kg, i.p.) or levetiracetam (50 mg/kg, i.p.), there was a significant improvement in the memory performance as indicated by discrimination ratios that were significantly higher than DFP exposed rats. Data expressed as mean \pm SEM, * p <0.05, t-test, n = 6 rats.

3.3. What opportunities for training and professional development has the project provided?

The GWIRP grant has allowed me to engage high school and undergraduate students in research and spark an interest in GWI research. My laboratory actively participates in VCU Summer Research Program. By informing the young students of GW history, sacrifices made by our Veterans, and how they can make a difference in the lives of GWI suffering Veterans by participating in our research program, my laboratory makes an attempt to get new generation of scientists interested in GWI research. This last part is especially true for the post-doctoral fellow working on this project and Medical school students who conduct summer-long research with us. The following students have conducted GWI-related research in 2016.

Name	Affiliation	Current Inst.
Ms. Shravani Wadwekar	Deep Run High School	VCU Bioinformatics
Mr. John Lucas	VCU Med School (M-2)	VCU M-3
Ms. Radhika Patel	VCU Med School (M-2)	VCU M-3
Ms. McKenzie Gray	VCU Health and Exercise Science	
Dr. Kristin Phillips	VCU Neurology	

3.4 How were the results disseminated to communities of interest?

Our work on the Gulf War Illness project was recently highlighted in the GWIRP program book. This program book is available on CDMRP website and is freely available to consumers and scientists at various DOD related meetings and seminar. We also presented our work on GWI model development and calcium level estimations to scientists and consumers at the 2016 Military Health System Research Symposium. Three peer-reviewed manuscripts were produced with the support of GWIRP grant. These manuscripts are freely available via VCU Scholars Compass and PMC Medline. We also actively promote our findings on social media such as Twitter, LinkedIn, and also on open-access scientific platforms.

3.5 What do you plan to do during the next reporting period to accomplish the goals?

In year 1 we established a rodent model of OP exposure that leads to the development of GWI related neurological morbidities by identifying DFP exposure levels. We also demonstrated the presence of chronic depression, anxiety, and memory impairments following such exposures and concurrent damage in related brain area. In year 2 we identified molecular mechanisms responsible for the expression of GWI psychiatric dysfunction by measuring intracellular Ca^{2+} levels using fluorescent microfluorimetry and estimating protein levels of components involved in the Ca^{2+} -induced Ca^{2+} release (CICR) machinery. In year 3, we will expand these studies to the 12-month time point and conduct pharmacological studies using agents that target components of CICR system in an attempt to identify therapeutic candidates for the effective treatment of GWI neurological morbidities.

4. Impact

The Research Advisory Committee on Gulf War Veterans Illness has strongly implicated exposure to OP nerve agents as leading cause for GWI. In the first year of this grant, my laboratory successfully developed a rodent model of OP exposure using the nerve agent

surrogate DFP. Rodents in this model exhibit chronic GWI symptoms reflecting the morbidities observed in Gulf War veterans including the development of anxiety, depression and cognitive deficits months after initial OP exposure. We have also identified neuronal damage in the same brain areas that have been reported to be compromised in GW veterans in clinical, functional and imaging studies. In the second year of this project we looked at molecular basis for the development of the neurological morbidities and for the first time report protracted elevations in intracellular calcium levels in GWI rats. We also report that alterations in calcium-induced calcium-release mechanisms are responsible for elevated calcium in GWI. Finally, using pharmacological agents that target these pathways we show that levetiracetam and dantrolene improve the symptoms of neurological morbidities in our GWI model. This research has offered new molecular targets for drug development and has identified two therapeutic candidates for the effective treatment of GWI neurological symptoms of depression, anxiety, and cognitive deficits.

4.1 What was the impact on the development of the principal discipline(s) of the project?

Our research using DFP exposures provides evidence that nerve agent exposure in the absence of other confounding factors such as stress, pyridostigmine tablets or other insecticides can produce neurological morbidities similar to GWI. We have also provided the first evidence for sustained elevations in intraneuronal calcium levels in GWI rats. Our research indicates that CICR plays a major role in mediating these prolonged neuronal calcium elevations. These calcium elevations could be lowered with dantrolene and levetiracetam treatment. Moreover, these drugs also reduced GWI neurological morbidities. This will have a major impact on the lives of veterans suffering from GWI by providing investigators a novel model of GWI symptoms to identify molecular bases of GWI in search of providing GW veterans with additional, effective therapeutic options.

4.2 What was the impact on other disciplines?

Exposure to OP agents that is occupational, accidental, or terrorism-related is a legitimate concern. Our work involving model development in year-1 has the capability to also serve as a rodent model of chronic OP exposure in the civilian population.

4.3 What was the impact on technology transfer?

“Nothing to report”

4.4 What was the impact on society beyond science and technology?

“Nothing to report”

5. Changes/ Problems:

We did not encounter any major problems during this reporting period. However, we faced technical difficulties while assessing CICR activity using bradykinin and caffeine stimulations. The neurons under manipulations drift off following agonist application making it difficult to complete the experiment and obtain statistically relevant data. After numerous technical adjustments, we feel we have optimized our experimental setup and are in the process of completing this set of experiments. We are also proposing to move the 3-month time point for behavioral analysis studies with U-73122 and H-89 to year 3 to coincide with 12-month assessment with these drugs. These drugs are very expensive and by testing them for the 2-

timepoints simultaneously, we can prevent drug waste, prevent shelf-life expiration and save significantly on the procurement costs.

6. Products

6.1 Publications, conference papers, and presentations

6.1.1 Journal publications:

1. Phillips K and Deshpande LS (2016) Repeated low-dose organophosphate DFP exposure leads to the development of depression and cognitive impairment in a rat model of Gulf War Illness. *Neurotoxicology*, 52: 127-33. PMID: 26619911.
2. Deshpande LS, Blair RE, Huang BA, Phillips KF and DeLorenzo RJ (2016) Pharmacological blockade of the calcium plateau provides neuroprotection following organophosphate paraoxon induced status epilepticus in rats. *Neurotoxicology and Teratology*, 56: 81-86. PMID: 27224207.
3. Deshpande LS, Blair RE, Phillips KF, DeLorenzo RJ (2016) Role of the calcium plateau in neuronal injury and behavioral morbidities following organophosphate intoxication. *Ann N Y Acad Sci*. 2016 Jun;1374(1):176-83. PMID: 27327161.

6.1.2 Other publications, conference papers, and presentations

1. Deshpande LS and Phillips K (2016) Elevated hippocampal calcium levels in an organophosphate-based rodent model of Gulf War Illness. MHSRS-16-0135, [Kissimmee, FL, Aug. 15-18 2016].
2. Deshpande LS (10-19-16) “Novel Molecular Mechanisms and Treatment Approaches for Neurological Morbidities in Gulf War Illness”. Invited Speaker Seminar Series at Department of Pharmacology and Toxicology, Brody School of Medicine, ECU Greenville, NC.

7. Participants & Other Collaborating Organizations

7.1 What individuals have worked on the project?

Name	Laxmikant Deshpande	Kristin Phillips	Robert Blair
Project Role	PI	Post-Doc Fellow	Investigator
Research Identifier	orcid.org/0000-0003-1491-1561		
eRA commons:	DESHPANDELS	KPHILLIPS5	REBLAIR
Nearest person month worked	6	10	2
Contribution to the project	DFP exposures, behavioral assays, estimation of calcium, data analysis and communication	DFP exposures, preparation of hippocampal slices, estimation of intracellular calcium	Protein isolation and estimations using western blotting
Funding support	DOD, NINDS	DOD	DOD, NINDS

7.2 Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

“Nothing to report”

7.3 What other organizations were involved as partners?

“Nothing to report”

8. Special reporting requirements

“Not applicable”

9. Appendices

9.1 Bibliography

9.2 Research highlights page from GWIRP program book

9.3 2016 MHSRS abstract and ECU presentation announcement

9.4 Peer reviewed publications cover pages

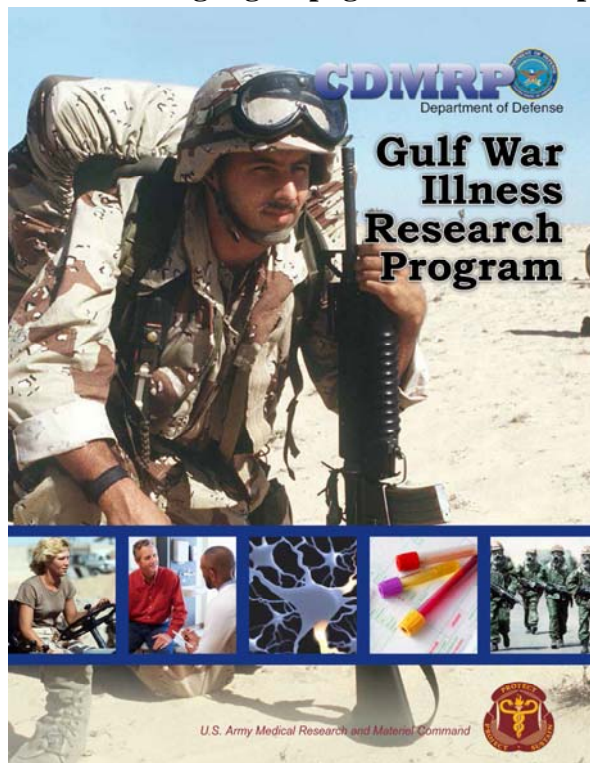
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9.2 Research highlights page from GWIRP program book



Novel Therapeutic Approaches for the Treatment of Depression and Cognitive Deficits in a Rodent Model of Gulf War Veterans' Illness

Dr. Laxmikant Deshpande, Virginia Commonwealth University



Dr. Laxmikant Deshpande used various (repeated low-dose to single high-dose) exposures to OP DFP over a 1- to 10-day period to approximate levels of sarin exposures during the Persian Gulf War. DFP-exposed rats were assessed for neurological impairments at 3 months post-exposure. Rats that were exposed to DFP exhibited symptoms of chronic depression, anxiety, and memory problems as characterized by increased immobility in the Forced Swim Test, anhedonia in the Sucrose Preference Test, anxiety in the Elevated Plus Maze, and spatial and recognition memory impairments in the Object Location/Recognition Test. Rats that were exposed to DFP experienced neuronal damage in the following regions – hippocampus, piriform cortex, amygdala, and thalamus – the same brain areas that have been reported to be compromised in GW Veterans in clinical, functional, and imaging studies. Taken together, these results are among the first evidence that nerve agent exposure, in the absence of other confounding factors such as stress, pyridostigmine tablets, or other insecticides, can produce neurological morbidities similar to GWI. The Principal Investigator's future plans include deciphering molecular mechanisms with particular emphasis on the role of intracellular calcium dynamics underlying the neuronal damage and neurological deficits in the DFP-exposed rats. Further, his plans also include addressing chronic/latent effects of exposure to GWI compounds with the inclusion of a 1-year assessment end point, and to study the effects of calcium-lowering drugs in an attempt to develop effective therapeutics that target the neurological abnormalities in his rat model.

9.3a MHSRS Abstract Details Abstract No. 16-0135

Title: Elevated hippocampal calcium levels in an organophosphate-based rodent model of Gulf War Illness

Authors: Deshpande, Laxmikant and Phillips, Kristin

Background: Around 35% of Persian Gulf War soldiers exhibit chronic multi-symptom illnesses also known as the Gulf War Illness (GWI). Amongst many causative factors, exposure to low-levels of Sarin has been strongly implicated for expression of GWI. Despite treatment recommendations, GWI veterans continue to suffer from anxiety, depression, and memory impairments. Thus there is an urgent need to identify molecular mechanisms underlying GWI and use this knowledge to develop effective GWI treatments. The role of calcium (Ca^{2+}) signaling in learning, memory and mood is well established. Disruptions in Ca^{2+} homeostasis are implicated in Alzheimer's, Parkinson's, TBI, and epilepsy. However, the status of Ca^{2+} homeostasis in the development of behavioral impairments in GWI is unknown.

Methods: Male Sprague-Dawley rats (8-weeks age) were exposed to organophosphate agent diisopropyl fluorophosphate (DFP, 400 $\mu\text{g/kg}$, s.c) over a 5-day period to approximate the duration and level of Sarin exposure during the Persian Gulf War. At 3-months post DFP exposure, depression and anxiety were evaluated using Sucrose Preference Test (SPT), Forced Swim Test (FST) and Elevated Plus Maze (EPM). Spatial memory deficits were evaluated in Object Location Test (OLT). For $[\text{Ca}^{2+}]_i$ estimation, acutely isolated hippocampal neurons were loaded with ratiometric Ca^{2+} indicator Fura-2AM and the resulting emissions were acquired.

Results: In the FST, GWI rats exhibited significantly increased immobility time (79.7 ± 11.5 s vs 37.7 ± 6.5 s) compared to age-matched control rats. In the SPT, GWI rats exhibited anhedonia-like condition (53.2 ± 4.8 % sucrose preference in GWI rats vs. 74.1 ± 4.1 % in control rats). GWI rats also displayed increased anxiety on the EPM (open-arm time: 29.3 ± 3.7 % control vs 9.4 ± 2.2 % GWI). In the OLT, GWI rats displayed spatial memory impairments (70.87% time at new location in control vs 48.7% in GWI, respectively).

Acutely isolated CA1 neurons from GWI rats manifested $[\text{Ca}^{2+}]_i$ of 399 ± 26 nM, that were significantly higher than $[\text{Ca}^{2+}]_i$ in neurons from age-matched controls (208 ± 16 nM, $n = 7$ animals, $p < 0.05$, t-test). Analysis of the population distributions of $[\text{Ca}^{2+}]_i$ revealed only 2% of age-matched control neurons exhibited $[\text{Ca}^{2+}]_i$ greater than 500 nM. In contrast, approximately 50% neurons isolated from GWI rats exhibited $[\text{Ca}^{2+}]_i$ between 250-500 nM and approximately 18% neurons exhibited $[\text{Ca}^{2+}]_i$ greater than 500 nM indicating a significant right-ward population shift towards higher Ca^{2+} concentration range ($p < 0.001$, Chi-square test, $n = 161$ neurons).

Conclusions: Here we observed depressive symptoms and cognitive deficits in rats exposed to chronic low-dose DFP. GWI rats manifested chronic elevations in hippocampal $[\text{Ca}^{2+}]_i$ and significant neuronal injury. Since Ca^{2+} is a major second messenger molecule, such sustained increases in its levels activate multiple signaling cascades, and alter gene expression of proteins involved in synaptic plasticity. It is plausible that these Ca^{2+} mediated alterations could underlie the delayed neuronal damage and long-term morbidity following DFP exposure. We are currently exploring the genesis of these protracted Ca^{2+} elevations and hypothesize that drugs targeted at lowering these Ca^{2+} increases could possibly be effective therapies for the treatment of GWI neurological symptoms.



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Elevated hippocampal calcium levels in an organophosphate-based rodent model of Gulf War Illness

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School of Medicine • Department of Neurology • Richmond, VA

MHSRS
16-0135

INTRODUCTION

- ~35% of Persian Gulf War soldiers exhibit chronic multi-symptom illnesses known as the Gulf War Illness (GWI).
- Exposure to low-levels of Sarin has been strongly implicated for expression of GWI.
- Despite treatment recommendations, GWI veterans suffer from anxiety, depression, and memory impairments.
- There is an urgent need to identify molecular mechanisms underlying GWI to develop effective GWI treatments.
- The role of neuronal calcium (Ca^{2+}) signaling in learning, memory and mood is well established.
- Disruptions in Ca^{2+} homeostasis are implicated in Alzheimer's, Parkinson's, TBI, and epilepsy which manifest neurological morbidities also seen in GWI.
- However, the status of Ca^{2+} homeostasis in the development of behavioral impairments in GWI is unknown.

METHODS

DFP exposure: Rats were injected with DFP (400 μ g/kg s.c. 1X) for 5-days, while control rats received saline injections.

Forced Swim Test (FST): FST was used to assess behavioral despair. Briefly, rats were forced to swim in a glass cylinder for a 5-min. Immobility, time spent in a glass cylinder, was recorded as a measure of behavioral despair. To keep it head above water rats were helped.

Sucrose Preference Test (SPT): SPT measures hedonic or lack of it by monitoring rat's preference to sucrose water. Rats had a free choice of either drinking 1% sucrose solution or plain water. Sucrose preference was calculated as percentage of plain volume of sucrose intake over the total volume of fluid intake and averaged over the 2 days of testing.

Elevated Plus Maze (EPM): EPM assess anxiety by taking into account the aversive behavior of rats to enter open-ended arms. Rats were tested in the EPM 1 hr after DFP exposure. The number of entries into the open and closed arms on EPM apparatus and behavior of each animal was recorded for 5 min.

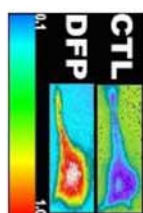
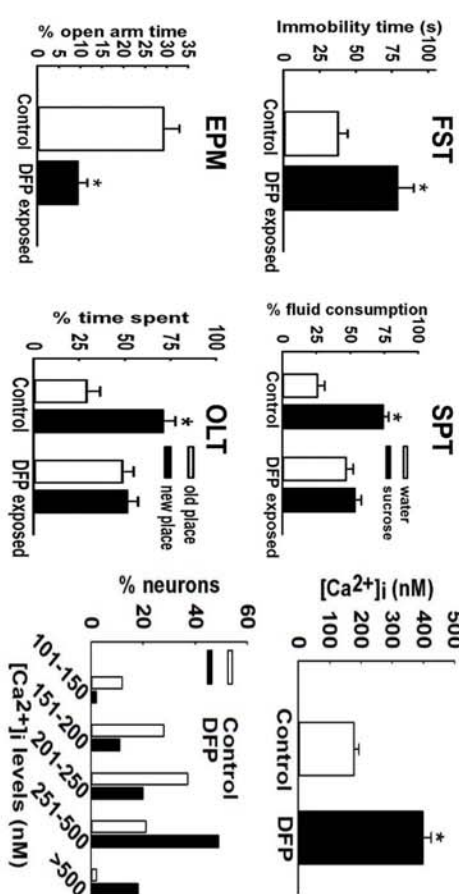
Object Location Test (OLT): This test assess place memory (spatial memory) by calculating the recognition of a rat to explore an object that has been moved to a new location.

Calcium Microfluorimetry: CA1 neurons were acutely isolated from hippocampal slices and loaded with fluorescent Ca^{2+} indicator Fura-2/AM. They were then stimulated using 400 nm light and Ca^{2+} transients as described earlier.

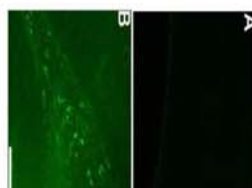
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1. Neurological morbidities in GWI rodent model



3. Neuronal injury



RESULTS

At 3-months following repeated low-dose DFP exposure over a 5-day period, rats were tested for neurological morbidities. We observed the presence of symptoms of chronic depression, anxiety and memory problems as characterized by increased immobility time in the FST, anxiety in the EPM, and spatial memory impairments in the OLT respectively (Fig. 1).

Acutely isolated CA1 neurons from GWI rats manifested [Ca^{2+}]_i of 399 ± 26 nM, that were significantly higher than [Ca^{2+}]_i in neurons from age-matched controls (208 ± 16 nM, n=7 animals, p<0.05, t-test). Analysis of the population distributions of [Ca^{2+}]_i revealed only 7% of age-matched control neurons exhibited [Ca^{2+}]_i greater than 500 nM. In contrast, ~50% neurons isolated from GWI rats exhibited [Ca^{2+}]_i between 250-500 nM and ~10% neurons exhibited [Ca^{2+}]_i greater than 500 nM, indicating a significant rightward population shift towards higher Ca^{2+} concentration range (p<0.001, Chi square test, n=161 neurons) (Fig. 2).

GWI rats also exhibited hippocampal neuronal damage as characterized by the presence of F-actin-positive staining in the polymorphic layer and along the hilar granule cell border of the dentate gyrus.

CONCLUSIONS

- Chronic depressive symptoms and cognitive deficits were observed in rats exposed to chronic, low-dose DFP. GWI rats manifested chronic elevations in hippocampal [Ca^{2+}]_i and significant neuronal injury.
- Since Ca^{2+} is a major second messenger molecule, such sustained increases in its levels activate multiple signaling cascades, and alter gene expression of proteins involved in synaptic plasticity. It is plausible that these Ca^{2+} mediated alterations could underlie the delayed neuronal damage and long term morbidity following DFP exposure in GWI rats.
- We are currently exploring the genesis of these protracted Ca^{2+} elevations and hypothesize that drugs targeted at lowering these Ca^{2+} increases could possibly be effective therapies for GWI neurological symptoms.

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9.4 Peer reviewed publications

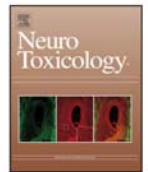
1. Phillips K and Deshpande LS (2016) Repeated low-dose organophosphate DFP exposure leads to the development of depression and cognitive impairment in a rat model of Gulf War Illness. *Neurotoxicology*, 52: 127–33. PMID: 26619911.

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NeuroToxicology



Repeated low-dose organophosphate DFP exposure leads to the development of depression and cognitive impairment in a rat model of Gulf War Illness



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ABSTRACT

Approximately 175,000–250,000 of the returning veterans from the 1991 Persian Gulf War exhibit chronic multi-symptom illnesses that includes neurologic co-morbidities such as depression, anxiety and cognitive impairments. Amongst a host of causative factors, exposure to low levels of the nerve agent Sarin has been strongly implicated for expression of Gulf War Illness (GWI). Nerve agents similar to pesticides are organophosphate (OP) compounds. There is evidence from civilian population that exposure to OPs such as in agricultural workers and nerve agents such as the survivors and first-responders of the Tokyo subway Sarin gas attack suffer from chronic neurological problems similar to GWI symptoms. Given this unique chemical profile, OPs are ideal to study the effects of nerve agents and develop models of GWI in civilian laboratories. In this study, we used repeated low-dose exposure to OP agent diisopropyl fluorophosphate (DFP) over a 5-day period to approximate the duration and level of Sarin exposure during the Persian Gulf War. We tested the rats at 3-months post DFP exposure. Using a battery of behavioral assays, we observed the presence of symptoms of chronic depression, anxiety and memory problems as characterized by increased immobility time in the Forced Swim Test, anhedonia in the Sucrose Preference Test, anxiety in the Elevated Plus Maze, and spatial memory impairments in the Object Location Test, respectively. Chronic low dose DFP exposure was also associated with hippocampal neuronal damage as characterized by the presence of Fluoro-Jade staining. Given that OP exposure is considered a leading cause of GWI related morbidities, this animal model will be ideally suited to study underlying molecular mechanisms for the expression of GWI neurological symptoms and identify drugs for the effective treatment of GWIs.

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1. Introduction

About 25–35% of the deployed soldier population from 1991 Gulf War suffer from a constellation of inexplicable symptoms referred to as Gulf War Illness (GWI). According to the Institute of Medicine's report, GWI also known as chronic multi-symptom illness is defined as the presence of a spectrum of chronic symptoms experienced for 6 months or longer in at least two of six categories: development of fatigue, mood and cognitive changes, musculoskeletal changes, gastrointestinal symptoms, respiratory difficulty, and neurologic abnormalities including major co-morbidities such as depression and anxiety ([Institute of Medicine: Board on the Health](#)

[of Select Populations, 2013](#)). There are several confounding factors attributed to development of GWI, including exposure to depleted uranium from tanks and body armor, prophylactic use of pyridostigmine bromide (PB) tablets, heavy use of insect repellants such as DEET and permethrin, smoke from oil-well fires, and dust particulate matter among others ([Friedl et al., 2009](#); [Steele et al., 2012](#); [Wolfe et al., 2002](#)). Interestingly, GWI symptoms have not been reported in veterans returning from other military conflicts suggesting that deployment-related stress is not a major factor in the expression of these multi-symptom illnesses ([Haley, 1997](#)). Newly assembled epidemiological, meteorological and intelligence data now indicate soldiers were exposed to organophosphate (OP) nerve agents Sarin and Cyclosarin from fallout released from demolitions of the ammunition dump at Khamisiyah, Iraq ([Couzin, 2004](#); [Haley and Tuite, 2013](#); [Special Assistant to the Secretary of Defense for Gulf War Illnesses, 2001](#); [Tuite and Haley, 2013](#)). After reviewing all the available data, the Research Advisory Committee

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2. Deshpande LS, Blair RE, Huang BA, Phillips KF and DeLorenzo RJ (2016) Pharmacological blockade of the calcium plateau provides neuroprotection following organophosphate paraoxon induced status epilepticus in rats. *Neurotoxicology and Teratology*, 56: 81-86. PMID: 27224207.

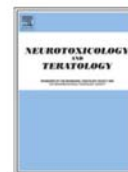
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Brief communication

Pharmacological blockade of the calcium plateau provides neuroprotection following organophosphate paraoxon induced status epilepticus in rats



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ABSTRACT

Organophosphate (OP) compounds which include nerve agents and pesticides are considered chemical threat agents. Currently approved antidotes are crucial in limiting OP mediated acute mortality. However, survivors of lethal OP exposure exhibit delayed neuronal injury and chronic behavioral morbidities. In this study, we investigated neuroprotective capabilities of dantrolene and carisbamate in a rat survival model of paraoxon (POX) induced status epilepticus (SE). Significant elevations in hippocampal calcium levels were observed 48-h post POX SE survival, and treatment with dantrolene (10 mg/kg, *i.m.*) and carisbamate (90 mg/kg, *i.m.*) lowered these protracted calcium elevations. POX SE induced delayed neuronal injury as characterized by Fluoro Jade C labeling was observed in critical brain areas including the dentate gyrus, parietal cortex, amygdala, and thalamus. Dantrolene and carisbamate treatment provided significant neuroprotection against delayed neuronal damage in these brain regions when administered one-hour after POX-SE. These results indicate that dantrolene or carisbamate could be effective adjuvant therapies to the existing countermeasures to reduce neuronal injury and behavioral morbidities post OP SE survival.

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1. Introduction

Organophosphate (OP) compounds are classified as lethal chemicals that include nerve gas and pesticides. Both the civilian and military population has been exposed to nerve agents under acts of war and terrorism (Haley and Tuite, 2013; Hood, 2001; Sellstrom et al., 2013). In addition, civilians are also exposed to OP compounds occupationally, intentionally (suicide) or due to accidents (Ajdacic-Gross et al., 2008; Konradsen, 2007; Than, 2013). Paraoxon (POX) is an active metabolite of parathion and is used in laboratory research to reliably model OP pesticide toxicity (Deshpande et al., 2014a). POX and other OP chemicals are potent inhibitors of the enzyme acetylcholine esterase (AChE) (Tuovinen, 2004). Inhibition of AChE prevents breakdown of acetylcholine (ACh) and rapidly builds up its level at the synapses. Overt stimulation of ACh receptors leads to the classical “cholinergic crisis” followed by respiratory depression, bradycardia and status epilepticus (SE). This prolonged seizure activity represents a clinical emergency and if left untreated results in the death (Bajgar, 2004). The current FDA approved OP treatment protocol involves administration of an anticholinergic drug atropine to manage hypercholinergic symptoms, an oxime

pralidoxime to reactivate AChE, and a benzodiazepine midazolam to stop SE (Chemical Hazards Emergency Medical Management, 2013). Despite the effectiveness of the standard three-drug regimen in limiting immediate mortality following OP exposure, OP/SE survivors are vulnerable to the development of chronic neurological morbidities (de Araujo Furtado et al., 2012; Deshpande et al., 2014b; Helmstaedter, 2007; Neligan and Shorvon, 2011; Rod, 2009; Phillips and Deshpande, 2016; Savage et al., 1988).

Our laboratory has developed SE survival models of OP toxicity using POX (Deshpande et al., 2014a; Deshpande et al., 2014b) and DFP (Deshpande et al., 2010). The mortality, behavioral manifestations and EEG profile for these OP SE models mimicked the signs and symptoms of human OP intoxication. Significant neuronal damage was observed throughout the limbic system in the brain of OP SE rats (Deshpande et al., 2014a; Deshpande et al., 2010). Subsequently, symptoms of chronic depression and memory impairments were also observed in these lethal OP exposed rats (Deshpande et al., 2014a; Deshpande et al., 2014b). These models provide a reproducible method to mimic the human survival of OP toxicity and are useful to screen novel medical countermeasures and also identify molecular mechanisms underlying the mortality and morbidity following OP intoxication.

One of the long standing interests of our laboratory has been studying role of Ca^{2+} homeostatic mechanisms following brain injuries (DeLorenzo et al., 2005). Ca^{2+} ions are major second messenger

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Issue: *Countermeasures Against Chemical Threats*

Role of the calcium plateau in neuronal injury and behavioral morbidities following organophosphate intoxication

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Organophosphate (OP) chemicals include nerve agents and pesticides, and there is a growing concern of OP-based chemical attacks against civilians. Current antidotes are essential in limiting immediate mortality associated with OP exposure. However, further research is needed to identify the molecular mechanisms underlying long-term neurological deficits following survival of OP toxicity in order to develop effective therapeutics. We have developed rat survival models of OP-induced status epilepticus (SE) that mimic chronic mortality and morbidity following OP intoxication. We have observed significant elevations in hippocampal calcium levels after OP SE that persisted for weeks following initial survival. Drugs inhibiting intracellular calcium-induced calcium release, such as dantrolene, levetiracetam, and carisbamate, lowered OP SE-mediated protracted calcium elevations. Given the critical role of calcium signaling in modulating behavior and cell death mechanisms, drugs targeted at preventing the development of the calcium plateau could enhance neuroprotection, help reduce morbidity, and improve outcomes following survival of OP SE.

Keywords: paraoxon; status epilepticus; cell death; calcium; dantrolene; carisbamate

The increasing risk for organophosphate exposure

Organophosphate (OP) chemicals include nerve agents, such as sarin, and pesticides, such as parathion. These compounds are considered extremely lethal. The civilian population has been exposed to nerve agents under acts of war and terrorism. Recent examples include the reported 2015 sarin gas attack in Ghouta, Syria,¹ the Tokyo subway sarin attack by the Aum Shinrikyo cult in 1995,² and the 1988 Halabja chemical attack against Kurdish people in Iraq.³ OP-based pesticides have also been used against civilians during the Rhodesian War,⁴ and Indian children were accidentally exposed following consumption of pesticide-contaminated lunches.⁵ In addition, civilians are exposed to OPs intentionally via suicide attempts, occupationally, or due to industrial accidents. In fact, pesticide ingestion is one of the most common methods for

committing suicide in developing nations.^{6–8} The military population has also been exposed to OP chemicals. Approximately 30% of Gulf War veterans suffer from a cluster of symptoms commonly known as Gulf War Syndrome. Prolonged exposure to OP-based pesticides or exposure to sarin gas following demolition of chemical weapon stockpiles is among the possible cause of this syndrome.^{9–11} The ease of availability of pesticides makes them attractive targets to weaponize and cause mass civilian casualties. Thus, there is a growing threat of OP toxicity in the current geopolitical environment. Research in this field has provided therapeutic antidotes that are critical in limiting immediate mortality associated with lethal OP intoxication.¹² However, further research is needed to identify the molecular mechanisms underlying chronic mortality and morbidity in order to develop effective counteract therapeutics following OP exposure.¹³